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ORIGINAL PAPER

Characterization of expressed genes in the establishment of arbuscular mycorrhiza between *Amorpha fruticosa* and *Glomus mosseae*

Fuqiang Song • Jize Li • Xingxing Zhang

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Abstract: Arbuscular mycorrhiza (AM) formed between plant roots and fungi is one of the most widespread symbiotic associations in nature. To understand the molecular mechanisms of AM formation, we profiled 30 symbiosis-related genes expressed in *Amorpha fruticosa* roots colonized by *Glomus mosseae* and in non-mycorrhizal roots at different stages using differential-display RT-PCR (DDRT-PCR). The expressed genes were confirmed by reverse Northern blotting. Eleven fragments were sequenced and putatively identified by homologous alignment. Of the eleven AM-related genes, five were obtained at the early-stage of plant-fungus interaction and six at the later stage. Three expressed sequence tag (ESTs) sequences were found to originate from the fungi and eight from the host plant by use of PCR evaluation of gDNA of both plant and fungi. The target genes included an ATP-binding cassette sub-family transporter gene, a transposon-insertion display band, and a photosynthesis-related gene. The results provided information on the molecular mechanisms underlying the development of mycorrhizal symbiosis between woody plants and AM fungi.

Keywords: arbuscular mycorrhiza, differential display, gene expression, *Glomus mosseae*

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Fuqiang Song (✉), Jize Li, Xingxing Zhang
Bio-ecological Restoration, School of Life Science, Heilongjiang University, 74#Xuefu Road, Harbin 150080, P. R. China.
Email: 0431sfq@163.com

Corresponding editor: Zhu Hong

Introduction

Amorpha fruticosa, a perennial leguminous woody shrub plant, is distributed mainly north of the Yangtze and Huaihe Rivers in China. It is an excellent shrub species for soil and water conservation and its roots, stems and leaves contain Amorphigenin, a flavonoid glycoside with a variety of medicinal properties, one of which is production of apigenin by hydrolysis. Because of its medicinal uses, the demand for *A. fruticosa* seedlings has increased gradually in recent years.

Arbuscular mycorrhiza (AM) is an ancient (>460 million years BC) symbiotic association between plants and soil fungi called AM fungi (Malloch et al. 1980; Brundrett 2002; Bonfante and Genre 2008). AM symbiosis is based on bidirectional nutrient exchange, i.e., AM fungi support the plants with nutrients such as phosphorus and nitrogen, and in turn they receive photosynthetic carbon from the plants (Harrison 1998; Ohtomo and Saito 2005; Paszkowsk 2006). In addition, the AM fungi can confer protection to the host plants against biotic (e.g., root pathogens) and abiotic stress (Vallino et al. 2008; Chen et al. 2009a; Evelin et al. 2009; Wehner et al. 2010). In nature, AM plays a significant role in biodiversity, evolution of land plants, ecosystem restoration and reconstruction, as well as agricultural, forestry and horticultural applications (Miransari et al. 2009; Hausmann and Hawkes, 2009; Uibopuu et al. 2009).

AM symbiosis is a result of complex and fine-tuned signaling events that lead to morphological and physiological alterations in both symbiotic partners. This process recruits genes from existing plant functions and modifies their expression patterns to fulfill the needs of AM formation. This may have resulted in the expression of mycorrhiza-regulated genes typical of higher plants and new genes with mycorrhiza-specific functions (Brechenmacher et al. 2004; Parniske 2004; Küster et al. 2007; Reinhardt 2007). Many genes related to symbiosis establishment have recently been isolated. The plant signaling network consists of three essential components, a receptor-like kinase, a predicted ion-channel and a calmodulin-dependent protein kinase, such as LjSYM2, ENOD11, LjSYMRK, PsSYM36, which regulate root

symbiosis (Kosuta et al. 2003; Grunwald et al. 2004; Kistner et al. 2005). In spite of this recent progress, a detailed morphological study of the symbiotic relationship is still needed. Being obligate biotrophs, AM fungi can not complete their life cycle without host roots, which hinder in-depth research on the signaling events in the establishment of symbiosis between fungi and host plants. To date, AM symbiosis research has focused on root nodule symbiosis (RNS) mainly in *Medicago truncatula* and *Lotus japonicus* (Antunes et al. 2006a). However, studies of mycorrhizal symbiosis in woody plants have rarely been reported. *Amorpha* is a genus of woody legumes, alfalfa plants, and lotus root, which can have different growth patterns and infection molecular pathways and can provide a theoretical basis for AM symbiosis research.

The transcriptome opens a window for insight into the symbiotic mechanism between AMF and *A. fruticosa*. Transcriptome, the total set of transcripts, is a widely used method nowadays, we can get thousands of papers in the database, so we did not describe it here. Previous research on symbiosis between AMF and A. fruticosa mainly on the physiology of the plant influenced by AMF, while transcriptome open the window for insight into the symbiotic mechanism between AMF and A. fruticosa. Much of our knowledge of AMF genetics, molecular biology and physiology is restricted to the species *G. intraradices*. The isolate DAOM197198 was chosen for the first genome sequencing project on AMF (Martin et al. 2008). The production of an assembled genome has proven to be an arduous challenge due to a gene space larger than expected, abundant transposons and a high level of polymorphism (Martin et al. 2008), as well as the difficulty of preparing sufficient pure genomic DNA. Initial sequence data (Martin et al. 2008) provided an indirect estimate of genome size of about 150 Mb, a value that has recently been confirmed experimentally (Sedzielewska et al. 2011). Nevertheless, the determination of the mitochondrial genome sequence of a *G. intraradices* isolate based on whole-genome shotgun sequencing, and the recent publication of the mitochondrial genome of two *Gigaspora* isolates (Pelin et al.; Nadimi et al. 2012), demonstrate that there is no longer any technical obstacle to obtaining sequence data from AMF.

In the absence of a complete sequence, our knowledge of the *G. intraradices* DAOM197198 genome has recently been expanded by the publication of genome-wide transcriptomic data (Tisserant et al. 2012). A rather large and highly diversified gene repertoire was inferred. The uniqueness of *Glomus*, and most probably of the AMF lineage, is reflected in the fact that 58.2% of the transcripts have no match in public nucleotide databases. A striking feature of the *Glomus* transcript data set is an abundance of sequence polymorphisms. This was not unexpected, since sequence variants have been described for a number of genes (Sanders et al. 2010), but the fact that these variants appear as expressed sequences implies the possibility of divergent functional roles.

Stable genetic transformation of AMF has not yet been achieved but Helber et al. (2008), recently used HIGS (host-induced gene silencing) as a tool to silence AMF genes expressed in planta. Since RNA silencing is based on the movement of RNA molecules, the success of HIGS with AMF raises the question of whether horizontal gene transfer events (HGT) have occurred at the plant–fungus interface, where there has been intimate contact between the partners for more than 400 million years. The parasitic plant *Striga hermonthica*, which forms an invasive organ called haustorium to allow transfer of nutrients form the host plant, recently provided an example of eukaryotic–eukaryotic HGT (Yoshida et al. 2010).

Materials and methods

AM fungi (AMF)

The AMF used in this study, *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe was obtained from the Chinese Academy of Agriculture Sciences (CAAS), Beijing, China. Inocula containing Sudan grass (*Sorghum sudanese* Staff) mycorrhizal root fragments, spores, and mycelia were obtained from a 1-year-old sterilized Sudan grass culture. The propagule was prepared with fungi suspension before inoculation. The concentration of the propagule in the suspension was about 80 spores/g-inocula for *G. mosseae.* All experiments were conducted in the Laboratory of Microbiology, Heilongjiang University, China (Song 2009).

Plant material

Amorpha fruticosa seeds were sterilized with 0.3% K_2MnO_4 for 20 min, and then germinated at 25°C for 60 h in an incubator after soaking for 24 h. The germinated seeds were then planted in pre-sterilized mixed matrix and grown under conditions of 25/18°C (day/night) temperature, 50% relative humidity, 14h photoperiod and 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density. The culture matrix was a mixture of peat soil, sand and vermiculite at a ratio of 5:2:3, was wrapped in brown paper and autoclaved at 121ºC, 0.1 MPa for 1.5 h, and then air dried one week before the start of the experiment. The plants were divided into two groups. One was inoculated with *G. mosseae,* whereas another was set as a control, i.e., inoculated with sterilized inocua (5% v/v). The inocula consisted of thoroughly mixed rhizosphere samples containing spores, hyphae and mycorrhizal root fragments. The plants in the greenhouse were collected after 14 days of growth and sampled every two days thereafter. Roots were washed extensively and carefully to separate them from the soil, quickly dried with paper, and either stained immediately, stored at 4°C for endophyte analysis or at -20°C for molecular analysis.

Root colonization analysis

Roots were stained with 0.12% cotton blue in lactic acid for about 20 h and then de-stained four times with lactic acid. The roots were cut into small pieces (1 cm each) and mounted onto microscope slides with lactic acid. For each root sample, 200 pieces were observed for a total of 600 root pieces. The intensity of fungal colonization into the root cortex and the presence of

arbuscules were determined as previously described (Trouvelot et al. 1986). Root fungal colonization was determined for each plant. Non-inoculated controls showed 0% AMF colonization in all cases.

RNA isolation

For the mRNA differential display analysis, root tissue from 3 biological replicates (*G. mosseae*- or mock-inoculated plants) were ground in liquid nitrogen using a preprocessed mortar and pestle. Total RNA was extracted from plant roots with AMF-colonized and non-colonized by Biozol using a RNA isolation kit which is Tiangen, Beijing, China. The RNA was suspended in a total volume of 100 μL double-distilled water. Agarose gel electrophoresis was used to test the integrity and a UV spectrophotometer was used to determine the purity of the RNA by monitoring the absorbance ratio at 260/280/230 nm.

The cDNA was synthesized from 5 μg of total RNA using the "First-strand Synthesis System for RT-PCR" kit and OligodT_{12}GC (TTTTTTTTTTTTGC). mRNA differential display was carried out as described in the Experiment Guide of Molecular Cloning (Sambrook et al. 1989; Liu et al. 2007; Fabi et al. 2009; Pandit et al. 2010). The anchored primers (H-T_{11} A: 5′-AAGCTTTTTTTTTTTA-3′, H-T_{11} G: 5′-AAGCTTTTTTTTTTTG-3′ and H-T_{11} C: 5′-AAGCTTTTTTTTTTTC-3′) were synthesized by Sangon, In a 0.5 mL micro-centrifugation tube, 2 μL of 10 × RT mixtures, 2 μL of 25 μM dNTP mixtures, and 2 μL of 10-μM Oligo-dT_{15} were added. The mixture was set to a volume of 19 μL by adding diethylpyrocarbonate-treated water. The tube was heated in a water bath at 37°C for 5 min, and then cooled on ice. Then, 0.5-μL total RNA and 1-μL Quant Reverse Transcriptase (200 U/μL) were added, maintained at 42°C for 60 min and then cooled on ice. The reverse transcription products were stored at 4°C for immediate use or at -20°C for later use. PCR amplification of the reverse transcription products was carried out in combination with one of the twenty arbitrary primers H-AP synthesized by Sangon (Table 1). The samples were denatured at 95°C for 1 min and then amplified as follows: 94°C for 15s, 45°C for 30 s, 72°C for 1.5 min for 40 cycles, and finally 72°C for 5 min. The amplified cDNA populations transcribed by 60 different mRNA pairs and one of the arbitrary primers with every anchored primer were size-fractionated by electrophoresis in 6% non-denaturing polyacrylamide gel. Silver staining of the polyacrylamide gel was performed according to the Bio-Rad Silver Stain Handbook.

Re-amplification

The gel bands of the differentially displayed cDNA fragments (DDF) were excised, diluted with sterile water, and boiled for 15 min. The eluted DNA was re-amplified in an Eppendorf tube using the same pair of primers as in the differential display. The PCR conditions were similar to those described above. The re-amplified DDFs were resolved using agarose gel electrophoresis and purified using the PCR DNA and Gel Band Purification kit, TIANquick Midi Purification Kit, Tiangen Biotech Co. Ltd., China.

Table 1: List of twenty arbitrary primers and three anchored primers

No.	Primers
B301	TACAACGACG
B302	TGGATTGGTC
B303	CTTTCTACCC
B304	TTTTGGCTCC
B305	GGAACCAATC
B306	AAACTCCGTC
B307	TCGATACAGG
B308	TGGTAAAGGG
B309	TCGGTCATAG
B310	GGTACATTGG
B311	TACCTAAGCG
B312	CTGCTTGATG
B313	GTTTTCGCAG
B314	GATCAAGTCC
B315	GATCCAGTAC
B316	GATCACGTAC
B317	GATCTGACAC
B318	GATCTCAGAC
B319	GATCATAGCC
B320	GATCAATCGC
B327	AAGCTTTTTTTTTG
B328	AAGCTTTTTTTTTA
B329	AAGCTTTTTTTTTC

Reverse northern blot

Probe labeling and detection of hybridization signals were carried out using DNA DIG labeling and DIG detecting kits, respectively, according to manufacturer protocols (Boeechringer-Mannheim Company, Germany). Sub-samples of 2 μL of amplified cDNA from the treated and control samples were denatured at 70°C for 5 min and then blotted onto a nylon membrane (Hybond-N^+, Amersham, UK). The DNA on the membrane between the two sheets of filter paper was fixed in an oven at 80°C for 2 h. Probe labeling and hybridization conditions were as described in the instructions of the Northern direct HRP labeling and detection kit, which is Biotin Chomogenic Detection Kit, MBI, Shanghai, China.

Cloning

Positive DDFs were cloned into a pMD18-T vector (Invitrogen), allowing blue-white selection in *Escherichia coli* DH-5α. Inserted DNA sequences of the plasmid clones were determined by automated sequencing by using ABI3330, Invitrogen, Shanghai, China. All sequences were aligned with the sequences in the non-redundant GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Verification of the origin of EST with PCR

Primers were designed based on the sequence information with Primer 3. Specific primers for the 11 differentially expressed ESTs were designed to give a total of 22 primers, of which Group 1 (AfSM1, AfSM3, AfSM6, AfSM7, AfSM9, AfSM10) had similar TM temperature, Group 2 (AfSM2, AfSM4, AfSM5, AfSM8) had similar TM values, and Group 3 (AfSM11) had the lowest TM values. The product sizes of AfSM1−AfSM11 were 192bp, 200bp, 205bp, 232bp, 253bp, 191bp, 183bp, 265bp, 192bp, 202bp and 202bp, respectively. Annealing temperatures were based on TMs during runs of the PCR program. Table 2 and Table 3 shows the amplification reaction system and amplification procedure of PCR.

Table 2: The amplification reaction system of PCR

Components	Volume
10×PCR buffer (Mg^{2+}Free)	2.5μL
50×Advantage cDNA polymerase	0.5μL
Mg^{2+}(25mmol)	1.5μL
dNTP（10μmol·L^{-1}）	0.5μL
PCR Primer 1（10μmol·L^{-1}）	1.0μL
PCR Primer 2（10μmol·L^{-1}）	1.0μL
Sterile H_2O	18.0μL
Total volume	25μL

Table 3: The amplification reaction procedure of PCR

Steps	Temperature	Time
Step1	94℃	10 min
Step2	94℃	1 min
Step3	54℃, 53℃, 51℃*	30 s

Notes: * The annealing temperature of Group 1, Group 2, Group 3 depended on their respective TM values.

Results

Mycorrhizal development

At different stages of inoculating *A. fruticosa* and *G. mosseae*, we selected 200 inoculated AMF roots samples for determining the extent of root fungal colonization using a microscope. The 0% of infection rate, no *G. mosseae* arbuscule, was observed in the root systems of *A. fruticosa* before the 15th day. Mycorrhizal plants had colonization levels of 12% at day 22, 31% at day 28, 53% at day 32, and 98% at day 35. AM development required time for colonizing. After colinization, symbiosis quickly took place. AM development can be divided into two stages, the early stage or initial 15 days, and the late stage, after 28 days, when colonization exceeded 30% (Fig. 1). We used these stages for isolating the symbiosis-related genes by differential display analyses.

The growth of inoculated (GM) was significantly better than for unvaccinated (CK) plants after culture in the greenhouse for 15 days (Fig. 2). Fig. 3 shows the vesicular and arbuscules of AMF colonized inside the roots at midanaphase colonization.

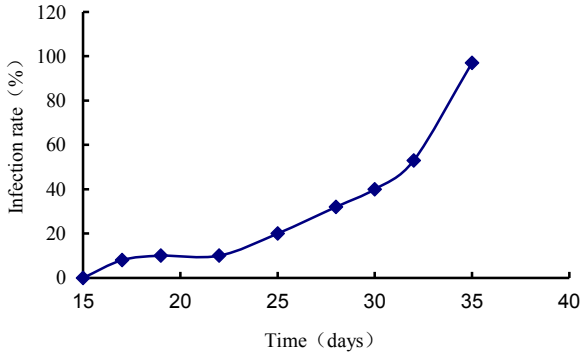

Fig. 1: Effect of seedling age on the colonization of arbuscular mycorrhiza fungi.

Fig. 2: Growth of GM and CK plants.

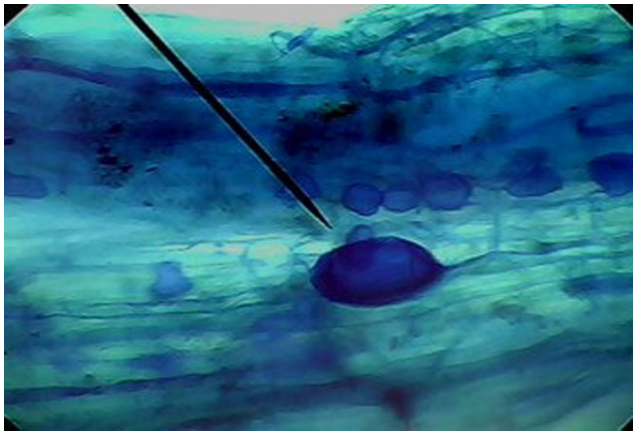

Fig. 3: The vesicular and arbuscules in the roots.

AM development

Total root RNA was extracted by Biozol and purified. The total RNA had high purity suitable for subsequent differential display

analysis.

In total, 60 primer combinations of 3 anchor primers and 20 arbitrary primers were amplified. These PCR products were separated on 6% non-denaturing polyacrylamide gels. Twenty to 50 bands were found in each lane. More differential fragments were found in the late stage (after day 28) than in the early stage (before day 14) (Fig. 4). Other samples had some bands representing both the early and late stages, and were expressed in pre- and post-mycorrhizal development. Some bands were obtained in the control but not in the samples of inoculated AMF, indicating that these genes may be associated with defense. Interestingly, some bands appeared or disappeared in the late or early stages of the symbiosis, indicating that the expression of the genes might stop and other genes might start to express at different stages. In addition, the expression of the same band may be strong or weak, indicating that gene expression levels vary at different stages of mycorrhizal formation.

Of the 12 positive ESTs, one (GW327873) did not show homologous sequences, whereas the other 11 ESTs can be divided into two types. One group (AfSM4, AfSM7, and AfSM9) was similar to bacterial proteins or fungi genes such as in *Penicillium* or *Ajellomyces capsulatus*, which may be vital for completing the fungal life cycle and the exchange of nutrients between *A. fruticosa* and *G. mosseae* at AM development. The other group (AfSM1, AfSM2, AfSM3, AfSM5, AfSM8, AfSM10, AfSM11, and AfSM12) was similar to plant proteins such as *L. japonicas* or *Ricinus communis* that may be specifically induced by the fungi and have an important role in AM establishment.

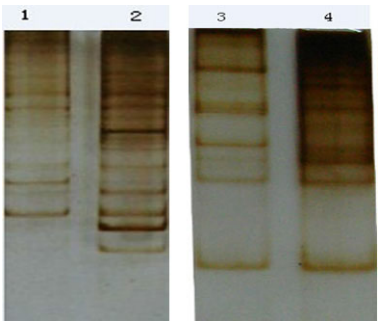

Fig. 4: Differentially expressed cDNA of agarose gel electrophoresis (1% agarose). Lanes 1 and 2 contain total RNA samples from control roots and *Amorpha fruticosa* colonized roots at early stage (14 days after infection), respectively; Lanes 3 and 4 contain total RNA extracted from control and colonized roots at late stage (28 days after infection).

DDRT-PCR of differentially expressed cDNAs from the samples

In total, we isolated 30 expressed fragment appearances Initial screening of the cDNAs to discard false positives was performed by reverse northern hybridization, and 12 fragments were isolated. Five of the 12 gene fragments were AM-related genes obtained from the *A. fruticosa-G. mosseae* in the early-stage interaction. Whereas, seven genes were isolated at the late stage of mycorrhizal development. Three ESTs corresponded to the origin of the fungi, whereas eight ESTs corresponded to plant genes. Putative functions were confirmed and sequence similarities were determined by Blastx analysis (Table 4).

Table 4: Sequence analysis of differentially expressed cDNAs by DDRT-PCR

Clone	Accession	Size (bp)	Identity (tBlastx)	Similarity	Evalue
AfSM1	GW317173	236	*Ricinus communis* hypothetical protein, mRNA	34/71 (48%)	9e-12
AfSM2	GW327877	312	*Lotus japonicas* class III HD-Zip protein	36/55 (65%)	2e-15
AfSM3	GW327876	354	ATP-binding cassette sub-family E member	35/38 (92%)	4e-13
AfSM4	GW327875	422	*Penicillium marneffei* ATCC 18224 conserved hypothetical protein, mRNA	44/108 (40%)	9e-19
AfSM5	GW327874	266	Hordeum vulgare subsp. vulgare respiratory burst oxidase-like protein B2 gene, complete cds	14/25 (56%)	6.7
AfSM6	GW327873	294	No match		
AfSM7	GW327915	350	*Flavobacterium johnsoniae* UW101, complete genome	15/31 (48%)	4.9
AfSM8	GW327914	372	Acetyltransferase, GNAT family protein	14/32 (43%)	1.1
AfSM9	GW327878	422	*Ajellomyces capsulatus* NAm1 conserved hypothetical protein (HCAG_06700) partial mRNA	40/101 (39%)	2e-17
AfSM10	GW327879	264	*Lotus japonicas* genomic DNA, chromosome 1, clone: LjT14B18, TM1868, complete sequence	34/71 (47%)	9e-12
AfSM11	GW327880	624	*Arabidopsis thaliana* clone SINE9 transposon-insertion display band genomic sequence	45/78 (57%)	8e-16

PCR results of the agarose gel electrophoresis (1% agarose)

Fig. 5A shows the consequence of gDNA of Amorpha fruticosa inoculated with AM fungi as a template, amplified by application six pairs of primers in Group 1. Fig. 5B shows the result of gDNA of Amorpha fruticosa without AM fungi as a template, amplified by application six pairs of primers in Group 1. Fig. 5C shows the consequence of gDNA of Amorpha fruticosa inoculated with AM fungi as a template, amplified by application four pairs of primers in Group 2. Fig. 5D shows the result of gDNA of Amorpha fruticosa without AM fungi as a template, amplified by application four pairs of primers in Group 2. Fig. 5E shows the consequence of gDNA of Amorpha fruticosa inoculated with AM fungi as a template in first lane and gDNA of Amorpha fruticosa without AM fungi in second lane, amplified by application primers in Group 3. The size of the marker was 2000 bp.

After amplification by PCR, the origins of AfSM 4, AfSM 7, AfSM 9 were determined to belong to *Glomus mosseae* as determined by evaluation of the photograph of agarose gel electrophoresis. The rest of expressed EST sequences were derived from Amorpha fruticosa. This consequence coincided with the results mentioned-above in Table 4, in which BLAST was used to search the EST sequences against GeneBank for functional annotation. The results also proved that the 3 different ESTs are important for fungi. We can also infer that AfSM 11 is derived from Amorpha fruticosa even though the band is not clear in Fig. 5E. This is because of the low expression or the annealing temperature.

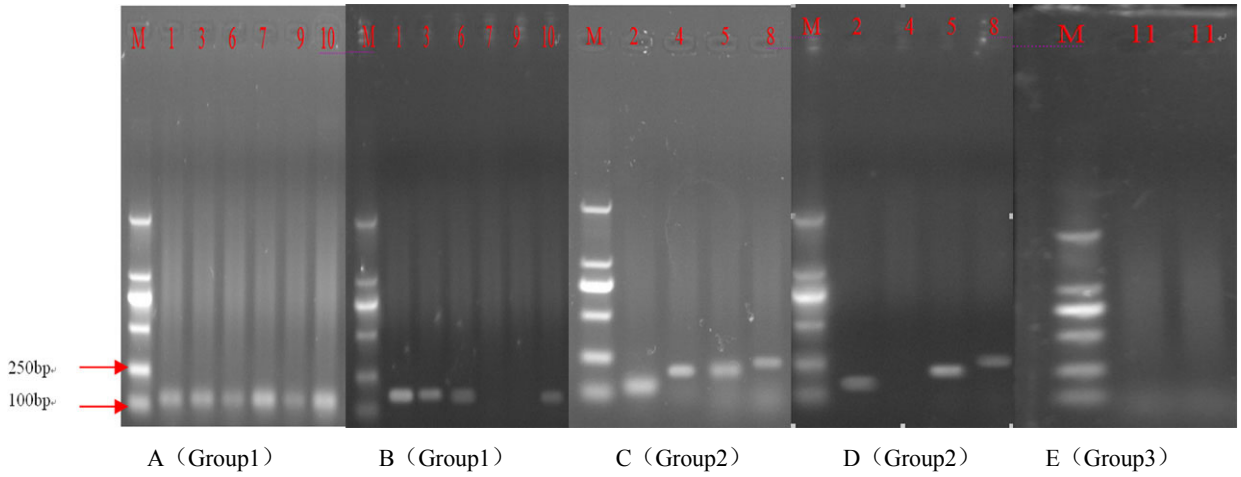

Fig. 5: PCR results of the agarose gel electrophoresis (1% agarose) to find the origins of 11 ESTs.

Discussion

In this study, the use of a well-controlled experimental mycorrhization system coupled with defined sampling times allowed us to attribute most of the 163 expressed genes to plant processes related to arbuscule development. After reverse northern hybridization, 11 genes were used to search for similarities to predict putative functions. For both symbionts, the period before the physical contact involved recognition and attraction of the appropriate partners and other events promoting mycorrhizal formation. The identified AfSM2 (*A. fruticosa* symbiosis) was similar to *L. japonicus* class III HD-Zip protein as it signals in response to external environmental stress and plays important regulatory roles in the unique developmental process of plants. The expression of AfSM2 is present both in the early and late stages, indicating that it plays an important role in the formation of AM. It is certainly an important mycorrhizal symbiosis-related gene that is required for increased AM fungi response to biotic and abiotic stress. AfSM3, similar to the ATP-binding cassette sub-family E member, belongs to a widespread transport system that moves metabolites, ions, sugars, amino acids, lipids, steroids, drugs and other substrates through the cell membrane. Many molecular components such as gas, sugar and secondary metabolites are transported across membranes so that plant and fungi can recognize each other for the pre-AM symbiosis (Fitter et al. 2011; Smith et al. 2011). AfSM4, similar to *Penicillium marneffei* hypothetical protein, is found mostly at the early stages. AM fungi form extensively branching hyphae called arbuscules in the cortical cells of the root due to the high levels of fungal gene expression (Bonfante and Genre 2010; Schüßler et al. 2010). AfSM5 is related to the respiratory oxidase gene and is highly expressed since there is a great increase in respiration and energy consumption at the early stage of mycorrhizal symbiosis (Krüger et al. 2012).

The similarity of AfSM8 with the acetyltransferase GNAT family protein indicates protein acetylation may be important for protein activity and structure. AMF can promote absorption by host plants of nutrients through the periarbuscular membrane in the mycorrhizal roots. Some protein transporters are used for exchange of N, P, and other minerals. These transporters are activated by acetylation (Lee et al. 2010; Rasmussen et al. 2008; Paungfoo-Lonhienne et al. 2010). AfSM7 and AfSM9, similar to the genes of *Flavobacterium johnsoniae* and *A. capsulatus* hypothetical proteins, might play an important role in the late stage of AM development to promote fungi-formed arbuscular structures in plant cells. These genes are connected to series genes to function in providing mineral nutrients for plants, and are expressed at the early stages to form hyphae (Mortimer et al. 2009; Nagy et al. 2009). AfSM10, similar to genes located in chromosome 1 of *Lotus japonicas* genomic DNA, may have a very specific function at the establishment of AM symbiosis. AfSM11, similar to *A. thaliana* clone SINE9 transposon-insertion display band genomic sequence, has been found to be important in the evolution of AM (Nadimi et al. 2012; Bonfante and Genre 2009). AfSM12, similar to a photosynthesis gene, synthetically constructs 'Red/Green ancestor' ancestral fluorescent protein variant D07 gene, and is expressed at AM formation (Dumbrell et al. 2010; Kaschuk et al. 2010).

Many scientists concur that AM, which has existed for billions of years, has an important role in the evolution of land plants in enhancing plant adaptations to their environments. However, little is known regarding the mechanism of mycorrhizal estab-

lishment and plant responses to adverse environments.

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